

EFFECT OF ETHIDIUM BROMIDE ON CYTOCHROME SYNTHESIS OF SV-40 TRANSFORMED CELLS

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1. Introduction

The utility of ethidium bromide (EB) and other dyes such as the acridines to study the development of mitochondria in yeast was first demonstrated by Slonimski and his collaborators [1, 2]. EB, which intercalates between bases of DNA [3, 4], is also known to differentially inhibit the synthesis and structure of mitochondrial DNA [5–7], activities of mitochondrial DNA polymerase [8], and RNA polymerase [9] in mammalian cells.

In yeast cells, EB is a powerful cytoplasmic mutagen [2, 5]. These cells lose their cytochromes aa_3 , b and c_1 , a change which is not reversible by omitting the drug.

In mammalian cells, however, none of the EB-induced effects reported were genetically stable. Human fibroblast cultures [11] and L cells [12] exposed to EB for various periods of time showed a reduction or loss of cytochrome aa_3 content. In L cells, however,

there is a concomitant increase in cytochrome c_1 and cytochrome c content [12], while the cytochrome c_1 synthesis is inhibited by treatment in yeast with EB as well as with chloramphenicol [13].

The present communication demonstrates that during 1 to 8 days of EB treatment on SV-40 virus transformed cell line, not only cytochrome aa_3 but also cytochromes b and c_1 reduce in their amounts with concomitant increase in the amount of uncharacterized b type cytochrome(s) absorbing at 557 nm at 77°K. When the cells were transferred to normal, drug-free medium these effects reversed; cytochromes aa_3 , b and c_1 increased with concomitant decrease in the amount of 557 nm-absorbing cytochrome(s). This report also deals with the cytochrome composition of an EB-resistant cell line, F [14], derived from F5-1 cells [15].

2. Materials and methods

2.1. Cells and culture methods

The SV-40 transformed cell line, F5-1 (passages 109 to 145), was derived from a primary tumor-bearing syrian gold hamster (Lakeview Colony) [15]. The F cells are a clone cell line derived from surviving cells of an F5-1 culture treated with 2 μ g EB/ml [14]. The cells were subsequently cultured in the presence of 2 μ g EB/ml for more than 1 year.

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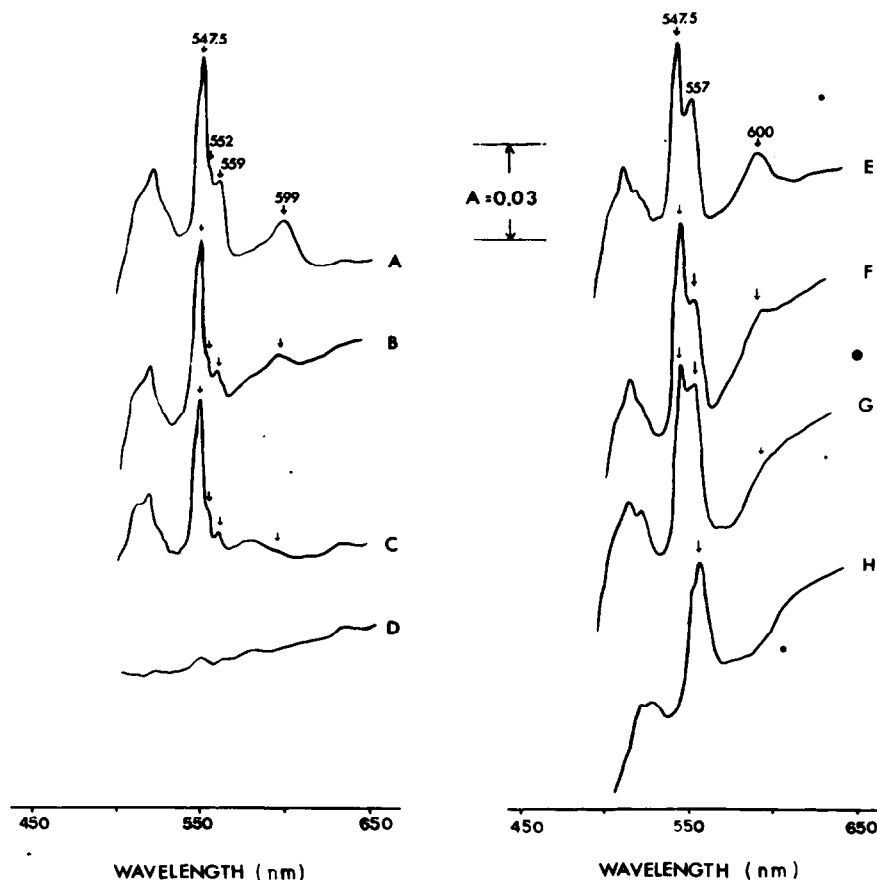


Fig. 1. The reduced-oxidized absorption spectra of the cytochromes of control and EB-treated F5-1 cells treated with $2 \mu\text{g}$ EB/ml for 4 days (spectra B and F), 8 days (spectra C and G), and 11 days (spectra D and H) which were suspended in phosphate-buffered saline at 57, 63, 50 and 15 mg protein/ml, respectively. Each reference sample was supplemented with $5 \mu\text{M}$ rotenone to oxidize the cytochromes in the respiratory chain, withdrawn 1 min later and injected into a spectrophotometer cuvette which had been precooled to the liquid nitrogen temperature [16]. In spectra A to D each measuring sample was treated with 5 mM KCN, withdrawn 1 min later and treated in the same way as the reference sample. In spectra E to H the measuring sample was treated with sodium-dithionite, withdrawn 2 min later and treated similarly. Light path, 2 mm. Temperature, 77°K .

All cells were maintained in Eagle's minimal essential medium supplemented with 10% fetal calf serum and $100 \mu\text{g/ml}$ streptomycin and 100 U/ml of penicillin.

The cells were harvested by the usual trypsinization procedure, suspended and washed twice with phosphate buffered saline [16] to remove phenol red in the preparation.

2.2. Determination of cytochromes

The absorption differences between the reduced and oxidized cytochromes at the temperature of liquid nitrogen were obtained in a split-beam

spectrophotometer [17]. In one experiment approximately 1×10^8 cells were suspended in phosphate buffered saline. The sample was frozen by the trapped steady state method [18]. The extinction coefficients of cytochromes, the absorbance change used and a conversion factor for the extinction coefficients at low temperature were given previously [10, 19].

3. Results

3.1. Spectral change induced by EB treatment

Approx. 1×10^7 F5-1 cells were seeded in Blake bottles (50 ml) in the presence of $2 \mu\text{g}$ EB/ml. Cell growth virtually ceased to retain almost the initial cell number during 8 days treatment. Fig. 1 illustrates the reduced minus oxidized spectra of cytochromes in control (A and E) and EB treated cells for 4 (B and F), 8 (C and G) and 11 (D and H) days. In spectra A to D the reduction of cytochromes was accomplished by adding KCN to aerobic respiring cells. The alpha region reveals the characteristic bands of cytochromes aa_3 , $b_{(K)}$ [20, 21], c_1 and c at 599, 559, 552 and 547.5 nm, respectively. When the cells were treated with EB, the absorbance of cytochromes aa_3 , b and c_1 decreases while that of cytochrome c remains unchanged up to 8 days of the treatment (spectra B and C). After 11 days of exposure, when half as many as the cells in the culture had died, the absorbance of cytochrome c as well as other cytochromes in the respiratory chain became extremely low.

In spectra E to H, where cytochromes were reduced by the addition of dithionite the absorbance of cytochrome aa_3 at 600 nm decreases after EB treatment. It is surprising that the b region of the spectra reveals an increased absorbance around 557 nm after EB treatment. In addition to cytochrome b in the respiratory chain [20–23] some other b cytochrome(s) seems to be involved. Cytochrome c_1 is not clear in spectra E–G even at the low temperature. No apparent change in the absorbance of cytochrome c can be seen at 4 and 8 days of exposure to EB. At 11 days when all the cytochromes in the respiratory chain became extremely low, only the absorption band of a b cytochrome(s) with an alpha maximum at 557 nm and a shoulder at 552 nm, a beta band at 528 nm and a Soret band at 424 nm (not shown) is seen. It is unlikely that the peak is attributable to the established b cytochromes. This b cytochrome(s) is tentatively designated as cytochrome b_{557} .

3.2. Change in cytochrome content following EB treatment

Table 1 summarizes the effect of EB on the cytochrome content in F5-1 cells. For 4 days following exposure to $2 \mu\text{g}$ EB/ml, the contents of cytochromes aa_3 , $b_{(K)}$ and c_1 decline at equal rates. After 6 days

Table 1
Cytochrome content during treatment of F5-1 cells with EB for various lengths of time.

Time of treatment (hr)	Cytochrome *				
	Cytochrome aa_3	Cytochrome b	Cytochrome c_1	Cytochrome c	Cytochrome b_{557} **
No treatment	9.3	11.8	5.4	32.2	10.3
24	8.8	8.3	4.9	33.4	9.3
48	5.9	7.3	3.7	30.8	9.2
96	3.1	3.6	2.5	32.3	14.1
144	1.4	3.5	2.4	28.2	23.2
192	0.8	3.4	2.5	32.7	33.6
264	n.d. ***	n.d.	n.d.	n.d.	87.0

* The F5-1 cells were treated with $2 \mu\text{g}$ ethidium bromide/ml for the length of time indicated. The cytochrome content, expressed in nmoles/g cell protein, was calculated from the spectra taken at 77°K .

** The content of cytochrome b_{557} was calculated from the difference spectrum of dithionite-treated cells minus KCN-treated cells using absorbance difference of 557 nm–575 nm.

*** Not determined.

of exposure, cytochrome aa_3 decreases significantly more than cytochromes $b_{(K)}$ and c_1 , amounting to only 10% of the control level at 8 days. The amount of cytochrome c , on the other hand, remains unaltered even at 8 days of exposure. At 11 days almost all the cytochromes in the respiratory chain have disappeared. Table 1 also shows that concomitant with the decrease of the amount of cytochrome aa_3 , the amount of cytochrome b_{557} is increased as much as 2–8 times the control level with 4 days lag time after the EB treatment.

When F5-1 cells were treated with chloramphenicol ($200 \mu\text{g}/\text{ml}$), the antibiotic affects the synthesis of respiratory cytochromes of F5-1 cells in a manner similar to yeast [24], algae [25], protozoa [26] and mammalian cells [27–29]. Cytochrome b_{557} remained unaffected during two days treatment and increased slightly (25%) in 4 days when the amounts of cytochromes aa_3 , b and c_1 decline to about 70% of the control level.

Table 2

Cytochrome content during recovery of F5-1 cells from treatment with EB

Time after treatment (hr)	Cytochrome*				
	<i>aa</i> ₃	<i>b</i>	<i>c</i> ₁	<i>c</i>	<i>b</i> ₅₅₇
Controls:					
A ¹	9.1	12.7	5.7	32.3	9.8
B ²	3.9	3.6	2.6	31.5	14.7
24	3.8	4.3	4.3	35.6	15.4
48	4.4	4.9	3.5	35.5	10.2
96	7.9	7.3	5.3	37.7	34.1
168	8.9	9.2	5.1	35.6	17.3

* Cytochrome content is expressed as nmoles/g cell protein. The spectra were taken at 77° K.

¹ Untreated F5-1 cells.

² F5-1 cells after 96 hr of treatment with 2 µg ethidium bromide/ml.

Table 3

Cytochrome content during recovery of F cells from treatment with EB.

Time after treatment (hr)	Cytochromes*				
	Cytochrome <i>aa</i> ₃	Cytochrome <i>b</i>	Cytochrome <i>c</i> ₁	Cytochrome <i>c</i>	Cytochrome <i>b</i> ₅₅₇
Controls					
A ¹	9.3	11.8	5.4	32.2	10.3
B ²	5.9	9.1	3.6	29.0	15.9
24	6.8	10.7	3.8	28.6	12.3
48	9.7	10.6	3.8	28.5	---
96	9.5	11.3	4.0	35.0	9.5

* Cytochrome content, expressed as nmoles/g cell protein. Spectra were taken at 77° K.

A¹: F5-1 untreated cells.

B²: F cells, resistant to 2 µg EB/ml, and cultivated at this concentration of the drug for 8 month.

3.3. Reversible effect on cytochrome content after omitting EB

To see whether the effect of EB is reversible or not Eb was omitted after 4 days of treatment. Inhibition of cell growth by EB treatment was released. The results are presented in table 2, which shows that in the presence of normal medium F5-1 cells are able to recover and yield normal amounts of cytochromes 4–6 days after EB treatment is discontinued. In 2–2.5 days half maximal synthesis of cytochromes *aa*₃, *b* and *c*₁ has been observed. The amount of cytochrome *c* is increased by approx. 30% during the course of recovery.

3.4. The cytochrome contents of EB-resistant F cells derived from F5-1 cells

The mitochondria of the EB-resistant F cells [14] appeared morphologically healthy. Analysis of cytochromes of these cells is presented in table 3. Cytochrome *aa*₃ decreased by 30%, *b* and *c*₁ both decreased by 20% and *c* remained almost at control level. When the F cells were transferred to normal culture medium containing no EB, cytochromes *aa*₃, *b* and *c*₁ increased to yield normal amounts in 2 to 4 days and cytochrome *c* content was slightly elevated.

4. Discussion

The results reported in this paper are completely in agreement with the inhibition observed on cytochrome *aa*₃ formation by EB in mammalian cells [11, 12, 29] and regenerating rat liver [32]. EB also inhibits the synthesis of cytochromes *b* and *c*₁ of F5-1 cells while the synthesis of cytochrome *c* is not inhibited. The inhibition of the synthesis of cytochrome *aa*₃ appeared more rapidly than the synthesis of cytochromes *b* and *c*₁. The synthesis of cytochrome *c* is finally inhibited by EB treatment. Since the initial cell number did not change significantly, the half life of cytochrome *aa*₃ might be estimated as 4 days (table 1).

Earlier workers did not demonstrate the inhibition of the synthesis of cytochrome *c*₁ [11]. This is presumably due to the use of dithionite as a reducing agent. The existence of multiple forms of cytochrome *b* [20–23], cytochrome *b*₅ or *b*₅-like pigment [30] often interferes with the differential determination of the amount of cytochromes *b*, *c*₁ and *c* in the respiratory chain. In the present investigation we used KCN to inhibit the electron transfer of mitochondrial respiratory chain of F5-1 or F cells, resulting in a complete reduction of respiratory cytochromes *c*, *c*₁ and *b*(K) (cf. [31]). For detection of all the cytochromes involved in the cells dithionite was used.

The *b* cytochrome(s) with an alpha band at 557 nm with a shoulder at 552 nm is spectrally different from cytochrome *b* in mammalian [21] and yeast [31] mitochondrial respiratory chain. This 557 nm-absorbing pigment(s) increased in its amount when cytochromes *aa*₃, *b* and *c*₁ decreased by EB treatment and decreased when the respiratory cytochromes were again increased in amounts by removal of the dye. The above data may support the idea that this cytochrome(s) is a modified form of cytochrome(s) in the respiratory chain. This pigment(s) is to be further characterized. The presence of similar *b* type cytochrome has been reported in anaerobic yeast [33], in ascites hepatoma cells [19] and in the SV-40 transformed cells (N. Sato, B. Chance, K. Kato and W. Klietmann, unpublished observation), in which the contents of cytochromes *aa*₃, *b* and *c*₁ are depressed as compared with those of the normal control.

The EB-resistant F cells show a significant difference in reaction to the drug from that of the parent F5-1 cells; the amount of cytochromes *aa*₃, *b* and *c*₁ appears to be considerably affected by EB, whereas cytochrome *c* is present in almost the same amount as in the untreated F5-1 cells. And yet the effect of long term EB treatment (1 year) is reversible when the cells are transferred to the normal EB-free medium. This result suggests that the mechanism of the resistance of F cells to EB might be partially due to a change in the composition of the cellular or mitochondrial membrane which causes a decrease in the amount of the dye that penetrates the cell or the mitochondria.

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